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Purification and N-Terminal Amino Acid Sequence of Two Birch Pollen Isoallergens (Bet v I and Bet v II)

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Abstract. The major allergen of birch pollen BV45 (Bet v I) was previously isolated by molecular weight exclusion chromatography and eluted in the molecular weight region of 15–29 KD. Further purification of this fraction on an SP-Trisacryl M cation exchange matrix allowed 6 peaks of which the 4th (BV4A4) and 6th (BV4A6) included two dominant IgE-binding birch pollen isoallergens designated Bet v I and Bet v II. Final purification, using the 'Applied Biosystems' Peptide Micro Separation System, revealed two sharp peaks with a high degree of homogeneity. This was ascertained by automatic N-terminal amino acid (AA) sequence analyses which showed high average repetitive yields of the phenyl-thiohydantoin (PTH) AAs of the isoallergens sequenced. N-terminal AA analyses of the two fractions allowed 51 cleavages with correct identifications of PTH AAs for 3 replicates. The sequence data of the two isoallergens showed large homologies with the hazel pollen allergen, Cor a I, the birch pollen allergen, Ag 23, and the translated cDNA sequence derived from cloning birch pollen allergen genes. The sequence homologies support that *Betula verrucosa* allergens were derived from a gene family expressing several isologous allergens, 2 of which with 13 variable residues in a segment of 51 AAs. The antigenicity of the two fractions, Bet v I and Bet v II, was demonstrated by fused rocket immunoelectrophoresis (FRIE) and by crossed immunoelectrophoresis (CIE) giving single symmetrical antigenic precipitation. The specific IgE-binding capacity of the two isoallergens was supported by: (a) immunoautoradiography of the plates provided from FRIE; (b) specific IgE-binding inhibition in radioallergosorbent test (RAST) for fraction BV4A, and (c) similarly high RAST inhibition for the individual isoallergens. The in vivo allergenic activity was clearly shown by skin prick and nasal provocation tests. In conclusion, two isoallergens from birch pollen with proximate pI values (5.2 and 5.5) were isolated for the first time and the N-terminal sequence characterized.

Introduction

A large number of proteins could be isolated from the pollen grains of Betulacea trees. Only a few of these have the capacity to bind specific IgE from the sera of atopic patients. The major allergens of hazel, alder and birch have been previously studied in details [1–5]. *Betula verrucosa* (BV) pollen allergens have been used in several laboratories as an adequate representative of tree pollen allergens. The major allergen, Bet v I [6], has been isolated and partially characterized [4, 5, 7, 8]. The N-terminal epitope residues No. 23–39 of this allergen have been prepared by solid phase peptide synthesis and its biological activity was studied in detail [9]. In several studies, two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the birch pollen antigens demonstrated a group of IgE-binding proteins with minor variations in their pI values [10]. The proximity of these pI values has probably hampered the isolation of these allergens in highly purified forms for many years. In this paper the isolation of two highly homogeneous isoallergens of birch and their N-terminal amino acid (AA) sequence will be precisely described for the first time.

Materials and Methods

Isolation and Purification of the Isoallergens

BV crude extract was produced as described before [5], using non-defatted pollen grains supplied by Allergon AB, Engelholm, Sweden. According to the manufacturer the pollen contains less than 2% w/w non-pollen debris and 0.5% w/w foreign pollens. The initial material BV4 produced by gel filtration chromatography (GFC) of the crude extract [5], was used for isolating two isologous proteins. Fraction BV4 encompassed Bet v I in addition to at least 10 other non-allergenic proteins [10]. Amounts of 40 mg BV4 protein were rechromatographed on the ACA-54 column (LKB, Bromma, Sweden), using 75 mmol/l sodium phosphate buffer as the mobile phase. The non-allergenic BV proteins were discarded. The allergenic fraction (BV4A) was pooled, dialyzed and lyophilized by the procedures previously described [5, 8, 10, 11]. Approximately 40 mg BV4A were applied on SP-Trisacryl M cation exchanger matrix (LKB, Bromma, Sweden, column dimensions 300 x 10 mm) using a 50-mmol/l sodium acetate buffer (pH 4.6) as the initial mobile phase, followed by a gradient buffer (pH 4.6–8.0) run in 60 m followed by a final mobile phase of 1 *M* NaOH. Fraction BV4A was resolved into 6 peaks, of which the 4th, BV4A4 (Bet v I), and the 6th, BV4A6 (Bet v II), were shown to bind IgE from allergic patients' sera.

Micro Separation System 130 A and a reversed phase (RP) 300 column (Applied Biosystem Inc., Foster City, Calif., USA) were used for the final optimal purification of the isoallergens Bet v I and Bet v II, using the readily available and standardized reagents supplied by the same manufacturer.

N-Terminal AA Sequence

The AA sequence of the entire chains of the two isoallergens, Bet v I and Bet v II, with tentative chain lengths of approximately 160 AAs [7], were performed using automatic Edman degradation (Applied Biosystem model 477 A). Protein/peptide sequencer and the phenylthiohydantoin (PTH) AA were analyzed on an on-line PTH-analyzer model 120 A (Applied Biosystem Inc., Foster City, Calif., USA). The average repetitive yields of the PTH AAs of the proteins sequenced were between 91.2 and 100%. The data from the PTH AA detector were converted to the computer by an interface (Nilson Analytical 900 series, Calif., USA). Otherwise the procedure described previously was utilized [12].

Immunoelectrophoretic Techniques

Antibodies against BV crude were raised in French Burgundy hybrid rabbits, locally bred at the animal colony of the University Hospital. The animal house is modern with excellent facilities and supervised by an authorized vet. The animals were sacrificed at the end of the process by legally accepted methods.

Crossed rocket immunoelectrophoresis (CIE), fused rocket immunoelectrophoresis (FRIE) and fused rocket radio-immunoelectrophoresis (FRRIE) were performed as described in detail in previous publications [3, 11, 13, 14]. For the first-dimension electrophoretic runs, 20 µg of the BV fractions were applied to wells in the primary gels. For the second-dimension CIE, 200 µl rabbit-anti BV (8 µl antibody/cm^2) were incorporated into the antibody gels. For the various FRIE runs, 11 µl of the investigated fractions were applied to the antigen wells and 700 µl anti-BV crude (15 µl/cm^2) were incorporated into the antibody gels.

The FRRIE procedure was performed by incubating the FRIE plates in a 6-fold dilution reaginic serum pool for 24 h at room temperature, followed by the washings and ^{125}I-rabbit anti-Fcε labelling. Unexposed Cronex 4 safety films (DuPont), calcium tungstate intensifying screens (Siemens High Speed) with exposures at –90°C were used [13]. The selected autoradiographic plates were evaluated after 12 h of exposure.

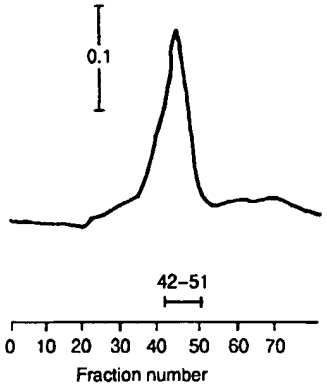

Fig. 1. The elution chromatogram of BV4 on an ACA 54 column showing the location of BV4A. The absorption range at 280 nm is indicated. These fractions were tested by RAST inhibition and the activities are shown in figure 7.

Human Sera

Reaginic sera from patients with evident clinical histories of tree pollen allergy, highly positive skin prick test (SPT) to alder, birch and hazel with radioallergosorbent test (RAST) class 3 to these allergens, were selected from the Allergy In vitro Diagnosis Unit, at the Central Laboratory, University Hospital. Ten sera were selected after informed consent of the patients, who donated these samples for research purpose. Sera were pooled in equal aliquots and stored at –20°C until used.

Specific IgE Inhibition Experiments

This was done by procedures described in earlier publications [5, 8–10]. Briefly, a serum pool from 10 birch pollen allergic patients was used in 2-fold dilutions. Aliquots of 100 µl of serum were incubated with an equal volume of BV4A fractions eluted from ACA-54 column (fig. 1). The reaction products were further used to bind the paper discs coated by the immobilized birch pollen allergens (Pharmacia Ab, Uppsala, Sweden). Otherwise the routine procedure used in our laboratory was followed [3].

In vivo Allergenic Activity of the Fractions

The biological activities of the isolated fractions BV4A4 (Bet v I) and BV4A6 (Bet v II) were investigated by SPT [3], nasal provocation tests and by Prausnitz-Küstner (PK) test. Wheal reactions were read after 20 min and the relative wheal area was deduced relative to 50 µmol/ml histamine chloride.

The nasal provocation tests were kindly performed by Sverre Steinsvåg, MD, at the Department of Otolaryngology, Head and Neck Surgery, University Hospital, by the procedures reported elsewhere [9].

As described earlier [11], the PK test was followed with serum

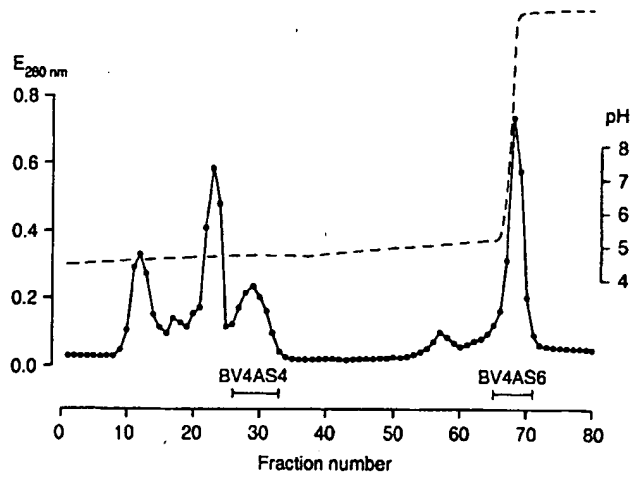

Fig. 2. Elution chromatogram of the cation exchange column. The chromatogram of BV4A on an SPTMC column, 300 × 10 mm, using an initial mobile phase 50 mmol/l CH_3COONa buffer. Repetitive runs of 40 mg BV4A dissolved in 1.5 ml start buffer (pH 4.6) were performed, and showed good reproducibility using a buffer gradient of pH 4.8–8.0 in 60 m.

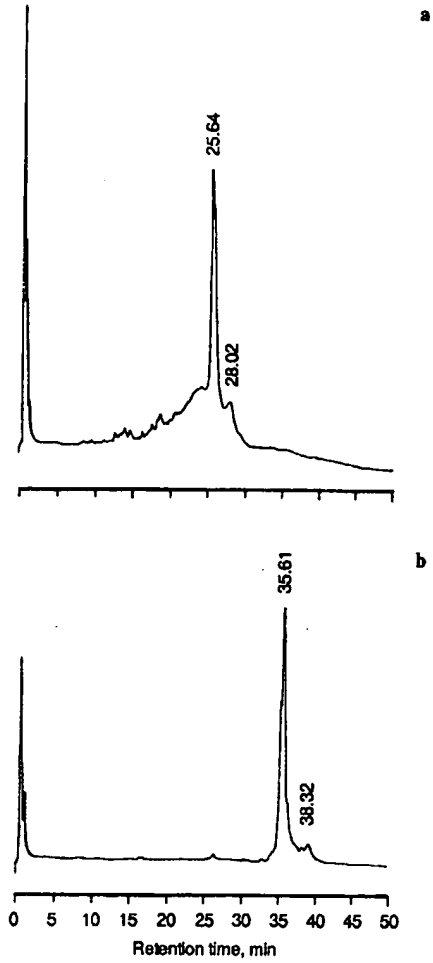

Fig. 3. High performance liquid chromatography purification. HPLC registration of fractions BV4 A4 (a) and BV4 A6 (b) showing two symmetrical peaks (AU 0.04) and confirming the presence of at least two isoallergens of the BV4A fraction. The isoallergens were eluted at different retention times.

taken from a healthy individual, not HIV or hepatitis-B infected. The serum was tested for both HIV and hepatitis B surface antigen. A negative serum sample was stored at –70°C for future use. A fresh blood sample was taken half a year later from the same donor and analyzed; the results were negative for viral antibodies. The 6-month-old stored serum of this donor was used on the arms of two healthy allergists (J.A. and H.V.) after the necessary informed consent from the donor and recipients had been received.

Results

Gel Permeation Chromatography

Fraction BV4, which was isolated in previous studies from this laboratory [15, fig. 1] by GFC, was intensively dialyzed, cutoff dialysis membrane of 8 KD, and selected for further purification. BV4 was rerun on an ACA-54 column, and the eluted fractions apparently encompassed the purified protein in fraction numbers 42–51, designated BV4A (fig. 1). The lyophilized BV4A which was further chromatographed on SP-1 column gave six UV-absorption peaks of which the fourth (BV4A4) and the sixth (BV4A6) were pooled and intensively dialyzed (fig. 2). The two fractions were optimally purified on the 130 A system (Applied Biosystem). Figure 3 confirms the degree of purification of Bet v I and II. For fraction Bet v I two extremely proximal peaks could be predicted, likely indicating the possible presence of 3 detectable isoallergens in birch pollen extract, one of them at a trace concentration.

AA sequence Analysis

The N-terminal AA sequences of BV4A4 and BV4A6 are presented in figure 4. The sequences showed large degrees of homology with the previously reported AA sequences for the major hazel pollen allergen Cor a I [1, 2], the birch pollen allergen Ag 23 [4] and with the translated cDNA sequence of Bet v I gene [7]. The assignment of more than 50 N-terminal AAs emphasized the extremely high homogeneity

1	2	3	4	5	6	7	8	9	10
G	V	F	N	Y	E	A	E	T	T(BV4AS4 890628)Bet v I
S	V	F	N	Y	E	T	E	T	T(BV4AS6 890403)Bet v II
G	V	F	N	Y	E	A	E	T	T (Hazel Cor a 1)
G	V	F	N	Y	E	A	E	T	T (Ag 23)
G	V	F	N	Y	E	T	E	T	T (cDNA sequence)

11	12	13	14	15	16	17	18	19	20
S	V	I	P	A	A	W	L	W	K(BV4AS4 890628)Bet v I
S	V	I	P	A	A	M	L	F	K(BV4AS6 890403)Bet v II
S	V	I	P	A	A	X	L	F	K (Hazel Cor a 1)
S	V	I	P	A	A	R	L	F	K (Ag 23)
S	V	I	P	A	A	R	L	F	K (cDNA sequence)

21	22	23	24	25	26	27	28	29	30
X	F	I	L	D	G	D	N	L	F(BV4AS4 890628)Bet v I
A	F	I	L	D	G	D	K	L	F(BV4AS6 890403)Bet v II
S	Y	V	L	D	G	D	K	L	L (Hazel Cor a 1)
A	F	I	L	D	G	D	N	L	F (Ag 23)
A	F	I	L	D	G	D	N	L	F (cDNA sequence)

31	32	33	34	35	36	37	38	39	40
P	K	V	A	P	Q	A	X	T	S(BV4AS4 890628)Bet vI
P	K	V	A	P	Q	X	Q	S	I(BV4AS6 890403)Bet vII
P	K	V	A	P	Q	A	L	T	S (Hazel Cor a 1)
P	K	V	A	P	-	-	-	-	- (Ag 23)
P	K	V	A	P	Q	A	I	S	S (cDNA sequence)

41	42	43	44	45	46	47	48	49	50
V	E	N	I	Y	E	R	G	G	W(BV4AS4 890628)Bet v I
V	X	N	X	Y	R	V	Y	X	P(BV4AS6 890403)Bet v II
V	E	N	V	G	G	N	X	X	P (Hazel Cor a 1)
K	E	N	N	P	W	L	T	A	Y (Ag 23)
V	E	N	I	E	G	N	G	G	P (cDNA sequence)

51	52	53	54	55	56	57	58	59	60
G	-	-	-	-	-	-	-	-	-(BV4AS4 890628)Bet v I
K	-	-	-	-	-	-	-	-	-(BV4AS6 890403)Bet v II
X	X	L/I	X	X	L/I				(Hazel Cor a 1)
	-	-	-	-	-				(Ag 23)
G	T	I	K	K	I				(cDNA sequence)

Fig. 4. Comparative N-terminal AA sequence of the isoallergens of birch and hazel. The two first sequences are those prepared in this study. The others were reported by Borch [1], Ipsen and Hansen [4] and Breiteneder et al. [7], respectively. Thirteen AAs at positions 1, 7, 17, 19, 28, 39, 40, 46, 47, 48, 50 and 51 were different between the two N-terminal segments of Bet v I and II.

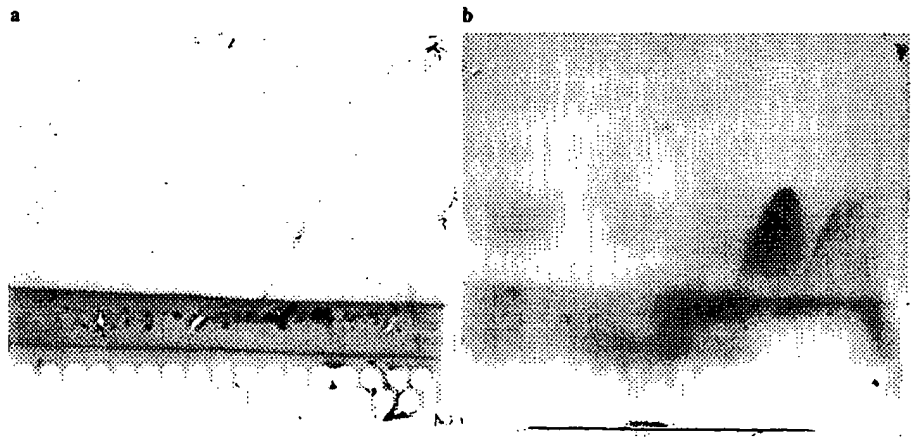

Fig. 5. Fused rocket immunoelectrophoresis and fused rocket radioimmunoelectrophoresis showing the uptake of ^{125}I rabbit anti-Fcε of the isoallergens isolated. The first precipitation peak corresponded to the fractions of Bet v I while the second one to Bet v II fractions.

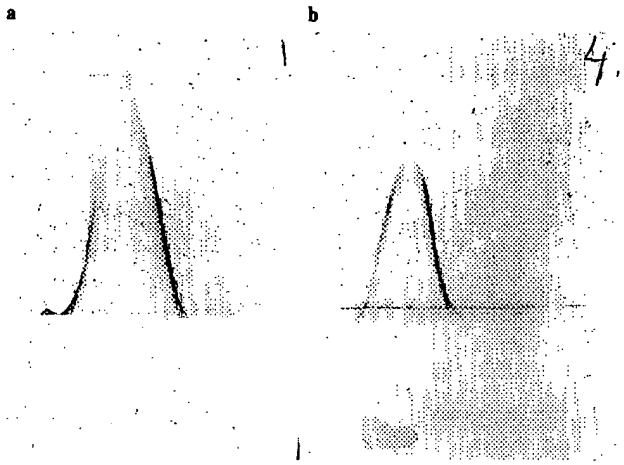

Fig. 6. Crossed immunoelectrophoresis of the isoallergens. CIE of the isoallergens BV4 A4 Bet v I (a) and BV4 A6 Bet v. II (b). One homogenous precipitance was found for Bet V II while that of Bet V I showed the presence of minor peak possibly of another isoallergen.

grade of the allergens isolated. These two isoallergens differed in 13 of 51 AA residues (G-1, A-7, W-17, W-19, N-28, T-39, S-40, E-46, R-47, G-48, W-50 and G-51), unraveling the difficulties experienced in earlier trials of isolating proteins with proximate pI variations. The percentage of the average repetitive yield for all the 51 analyzed PTH AAs were more than 90% and variances with values mostly close to 1.00. The average and combined AA repetitive yield was between 92.8 and 100% (raw material not shown). The sequence analysis for each protein was at least reproduced by three replicates with similar average and combined repetitive yields as mentioned.

Antigenic Activity

The antigenicity of BV4 fractions were confirmed by FRIE as shown in figure 5. The precipitation lines were located at the wells corresponding to fractions No. 28–35 of BV4A4 and No. 64–72 of BV4A6 (fig. 5a). These antigenic lines could react with specific IgE in the patients' sera (fig. 5b). The antigenicity of BV4A4 (Bet v I) and BV4A6 (Bet v II) was thus encompassed in BV4. Furthermore, the antigenicity of fraction Bet v I and Bet v II was examined by CIE; the results showed one dominant and a trace of another antigen precipitate for Bet v I, while for Bet v II only one antigenic precipitation was visualized (fig. 6). These lines could bind reaginic IgE and ^{125}I rabbit anti-Fcε as demonstrated by corresponding autoradiography.

Allergenic Activity

The allergenicity of BV4A eluted from the ACA-54 column was tested by specific IgE inhibition (fig. 7). The IgE-binding capacity of the eluted protein BV4A was demonstrated by their high inhibition percentage (range 62–95.7% with maximum inhibition of 95.7% at fractions 47, 48 and 58). RAST inhibition was similarly performed on the isoallergens. Both showed an inhibition of birch pollen allergen discs of > 90%. The in vivo allergenicity of BV4A4 (Bet v I) and BV4A6 (Bet v II) at the concentration 150 nmol/ml were investigated in SPT in 2 birch-allergic patients of which the results from patient B.R. is presented in table 1. For both patients wheal reactions, approximately two times larger than that of the 50-μmol/ml histamine control, were observed. Two non-allergic volunteers showed no SPT reactions to the investigated material.

Nasal provocations were performed using both the

initial preparation BV4A, Bet v I and Bet v II at concentrations of 150 nmol/ml. For the 2 allergic patients tested, B.R. and T.G., no reactions were observed during the anesthesia procedure or by provocation with a physiological saline solution. Itching, sneezing and rhinorrhea were registered less than 20 s after the application of the BV allergens. On inspection rubor and edema appeared round the nose and secretions were seen from the ipsilateral eye. For patient T.G. an additional partial ipsilateral nasal stenosis was found. For the 2 controls no clinical reactions or alterations of the mucous membranes were seen.

In direct PK tests, resactions to 2 histamine equivalent pricks for either of Bet v I and Bet v II were obtained in the skin sites of the volunteers described in Methods. For the PK inhibition specific IgE serum was incubated with Bet v I prior to intracutaneous injection. This resulted in a remarkably reduced wheal reaction upon provocation of the sites with the same antigens 24 h later.

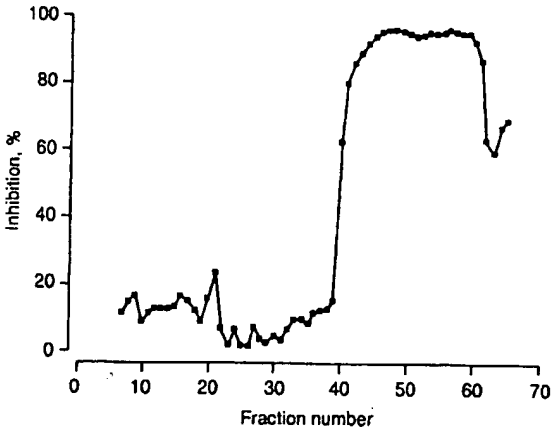

Fig. 7. The in vitro allergenic activity of the isoallergens. Specific IgE-inhibition diagram of BV fractions eluted from SPTMC. The IgE binding of the isoallergens was demonstrated by their high inhibition percents (range 62–95.7%) with the maximums at fractions 47, 48 and 58.

Discussion

The major allergens of birch and other Betulaceae family represent a group of isoallergens which are extremely related in their protein primary structures and are coded by a family of very similar genes [16]. GFC of the birch pollen crude extract resulted in a dominant IgE-binding material and BV4 was isolated [5]. Refractionation on a similar GFC column resulted in fraction BV4A (fig. 1). The range of inhibition of these fractions was between 62 and 95.7% (fig. 6). BV4A was further purified by the cation exchange chromatography where six peaks were resolved. Two of these, BV4A4 (Bet v I) with a pI of 5.2 and BV4A6 (Bet v II) with pI 5.5, included two birch IgE-binding proteins. RP chromatography showed two major isoallergens and one in trace amounts. Both the two dominant isoallergens have different retention elution times from the RP column (fig. 3). N-terminal AA sequence analyses of the fractions demonstrated a very high degree of purity isoallergens, allowing automatic determination of 51 N-terminal AA (fig. 4). The N-terminal analysis was performed under controlled procedures with reliable automatic instrumentation, leaving no doubt about the correctness of the N-terminal sequence of these two isoallergens. Bet v II was demonstrated to be the most homogeneous as judged by the sequence data, CIE pattern and RP HPLC registration. The pI value differences indicated the presence of birch pollen isoallergens with only three charged AA replacements. The homology with Ag 23 [4] and with the major allergen of hazel pollen Cor a I [1, 2] confirmed these observations.

Table 1. In vivo allergenicity of the two isoallergens

Allergen used	Concentration nmol/ml	Mean wheal diameter, mm	Histamine equivalent
BV4A	150	13.25	2.65
BV4AS4 (Bet v I)	150	9	1.8
BV4AS6 (Bet v II)	150	9.75	1.95
BV	150	11	2.2
Histamine chloride	$5 \cdot 10^4$	5	

SPT wheal reactions towards BV4A, Bet v I and Bet v II as compared to BV crude extract and histamine chloride reaction in 1 birch-allergic patient (B.R.).

The antigenicity of the isoallergens were also demonstrated by the immunoprecipitates obtained against polyclonal rabbit antibodies against BV extract.

The in vitro allergenicity of Bet v I and Bet v II were demonstrated by the specific IgE binding in RAST inhibition and by radiostaining in CRIE. The

described in vivo allergenic activities in PK investigations and nasal provocations of 2 birch allergic patients confirmed the allergenic activities of the isoallergens.

In conclusion two isoallergens of the major birch pollen allergen, Bet v I and Bet v II, with proximate pI values are described for the first time. The high degree of homogeneity of the two isoallergens was indicated by performing more than 51 N-terminal AA residues of the chains and by the high average repetitive yields of the different protein sequences. The antigenicity and allergenicity of the two isoallergens were clearly demonstrated both in vivo and in vitro.

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